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Hydrogen production by fermentative consortia

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ABSTRACT

In this work, H_2 production by anaerobic mixed cultures was reviewed. First, the different anaerobic microbial communities that have a direct relation with the generation or consumption of H_2 are discussed. Then, the different methods used to inhibit the H_2-consuming bacteria are analyzed (mainly in the methanogenesis phase) such as biokinetic control (low pH and short hydraulic retention time), heat-shock treatment and chemical inhibitors along with their advantages/disadvantages for their application on an industrial scale. After that, biochemical pathways of carbohydrate degradation to H_2, organic acids and solvents are showed. Fourth, structure, diversity and dynamics of H_2-producers communities are detailed. Later, the hydrogenase structure and activity is related with H_2 production. Also, the causes for H_2 production inhibition are analyzed along with strategies to avoid it. Finally, immobilized-cells systems are presented as a way to enhance H_2 production.

Contents

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Abbreviations: AC, activated carbon; ALSC, acrylic latex plus silicone; ATP, adenosine triphosphate; BES, 2-bromoethanesulfonate; CoA, coenzyme A; COD, chemical oxygen demand; *Cp*I, bidirectional hydrogenase from *Clostridium pasteurianum*; *Cp*II, H_2-oxidizing hydrogenase from *Clostridium pasteurianum*; CSTR, continuous stirred tank reactors; *D*, dilution rate; DGGE, denaturing gradient gel electrophoresis; DNA, deoxyribonucleic acid; E_A, activation energy; EC, expanded clay; HRT, hydraulic retention time; HSP, heat-shock pretreatment; IV-SSAH, intermittently vented solid substrate for anaerobic H_2 generation; LAB, lactic acid bacteria; NADH, reduced nicotinamide adenine dinucleotide; NRB, nitrate-reducing bacteria; OFMSW, organic fraction of municipal solid waste; PCR, polymerase chain reaction; PU, polyurethane; rRNA, ribosomal ribonucleic acid; SEM, scanning electron microscopy; S_0, substrate concentration; SRB, sulfate-reducing bacteria; T-RFLP, terminal restriction fragment length polymorphism; TS, total solids; X_0, initial sludge density.

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1. Introduction

For decades, the use and abuse of fossil fuels (either in liquid, gaseous or solid form) has caused contamination of our soil, air and water. Recently, diverse alternative fuels have been proposed to substitute fossil fuels. Hydrogen is one of these alternative fuels that is recognized as a promising future energy carrier. It is considered a clean fuel since it does not have carbon, sulfur or nitrogen that cause pollution during combustion [96]. Today, H_2 is principally produced from fossil fuels such as natural gas and naphtha. However, this practice is an environmental contradiction since a clean fuel is generated from a polluting and limited source. Therefore, it is necessary to use other sources and methods to obtain H_2 in a renewable, sustainable and environmentally friendly way.

In this regard, biotechnology can provide H_2 from renewable, cheap and abundant sources such as wastewater or organic solid wastes. This way, the use of pure cultures of anaerobes, aerobes, photosynthetic bacteria and cyanobacteria have been reported with the objective of generating H_2 [60]. Also, it is possible to use undefined microbial consortia to generate H_2 from a fermentation process. The use of fermentative consortia presents several advantages such as high H_2 generation rates (~100 times more than with photosynthetic cultures), continuous H_2 generation at a sustained rate since it does not depend on light energy as a photosynthetic process does, generation of metabolites of commercial interest (such as organics acids and solvents), oxygen limitation does not exist because it is an anaerobic process and the most important fact is it can use complex organic waste as a substrate in non-sterile conditions [13,46,45]. The use of organic wastes instead of pure carbohydrates is the main advantage of the fermentative process utilizing consortia since the costs for implementation to full scale is smaller. Thus, it is possible to generate H_2 from wastewater or municipal/industrial/agricultural solid wastes, avoiding their incineration or disposal in landfills. In spite of their potential, this technology has not been studied much and there are still many limitations to overcome.

The scope of this review is to present an updated perspective presenting more than 90 publications that are direct or indirectly related with H_2 production by fermentative consortia. This search was focused on (i) H_2 evolution in anaerobic environments; (ii) induction of H_2 accumulation by biokinetic control, heat-shock treatment and chemical inhibitors; (iii) structure, diversity and dynamics of H_2-producers communities; (iv) basic biochemical aspects such as the metabolic pathways of carbohydrate anaerobic degradation into H_2; (v) hydrogenases related with H_2 evolution, focusing on conditions that affect their activity; (vi) inhibition of H_2 production by products such as organic acids/solvents and H_2 as well as the methods used to prevent that inhibition (gas sparging, membranes).

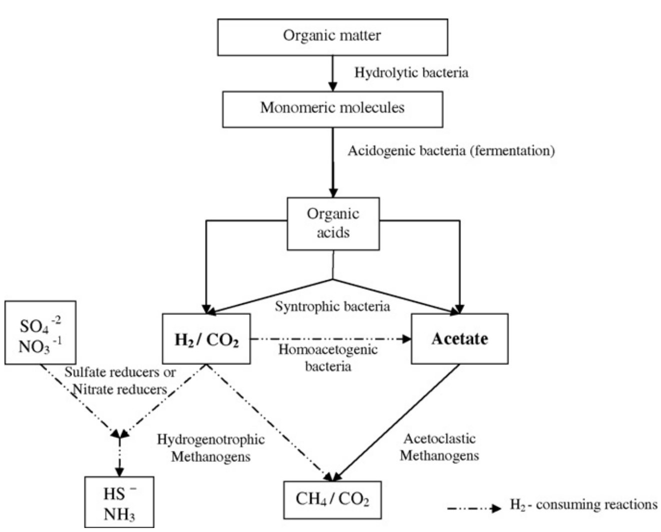


Fig. 1. Hydrogen role in the anaerobic degradation of organic matter by microbial consortia. Adapted from [23].

2. Hydrogen: a key intermediate in anaerobic environments

The degradation of organic matter in anaerobic environments by microbial consortia involves the cooperation of a population of microorganisms that generate a stable, self-regulating fermentation [81]. First, hydrolytic bacteria hydrolyze polymeric proteins and sugars. Then, fermentative bacteria form organic acids, H_2 and CO_2 from monomeric molecules (Fig. 1). At that point, H_2 and acetate can be utilized and/or produced by several microbial groups. Thus, acetate is generated during acetogenesis from CO_2 reduction and H_2 by autotrophic acetogens via the Wood–Ljungdahl pathway, a process named homoacetogenesis [57]. Also, synthophic bacteria generate acetate along with additional H_2 from short-chain organic acids (except acetate). Finally, for a complete degradation of organic matter, the consumption of organic acids and H_2 by acetoclastic/hydrogenotrophic methanogens producing CH_4 and CO_2 is essential [23]. In addition, when sulfates or nitrates are present, sulfate-reducing bacteria (SRB) and nitrate-reducing bacteria (NRB) are capable of using H_2 as electron donors generating sulfides and ammonia, respectively (Fig. 1).

Thus, H_2 is a key intermediate consumed mainly by methanogens, NRB, SRB and homoacetongens. The H_2 consumption enables biochemical reactions carried out by syntrophic bacteria (Table 1) to become exergonic and syntrophs can produce additional H_2 from organic acids [83]. This obligatory association between H_2-producing and H_2-utilizing microorganisms is called syntrophy. In consequence, H_2 concentration and the activity of H_2-utilizing microorganisms may regulate the fermentative pathways. Due to a rapid H_2 consumption, their concentration is usually extremely low and microorganisms have to compete for it. Therefore, establishment of one type of H_2 consumer depends mainly on the type of inoculum, H_2 concentration, carbon source, solubility of electron acceptor and capacity to utilize H_2 traces.

Studies have demostrated that under the best growth conditions for all H_2 consumers, the major capacity to utilize H_2 traces is related to a more energetically favorable biochemical reaction [12].

Table 1
H_2-producing and H_2-consuming reactions presents in anaerobic processes

Equations	Type of reaction	Reaction	Gibbs free energy (kJ/reaction)	
			ΔG° (a)	$\Delta G^{\circ\prime}$ (b)
1	Fermentation	$C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + \text{butyrate} + 2HCO_3^- + 3H^+$	−135	−284
2	Fermentation	$C_6H_{12}O_6 + 4H_2O \rightarrow 4H_2 + 2\text{acetate} + 2HCO_3^- + 4H^+$	−207	−319
3	Anaerobic oxidation (syntrophy)	$\text{Butyrate} + 2H_2O \rightarrow 2H_2 + 2\text{acetate} + H^+$	+48.2	−17.6
4	Anaerobic oxidation (syntrophy)	$\text{Propionate} + 3H_2O \rightarrow 3H_2 + \text{acetate} + HCO_3^- + H^+$	+76.2	−5.5
5	Hydrogenotrophic methanogenesis	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−136	−3.2
6	Acetogenesis from CO_2 and H_2	$4H_2 + 2HCO_3^- + H^+ \rightarrow \text{acetate} + 4H_2O$	−105	−7.1
7	Sulfate reduction	$4H_2 + SO_4^{-2} \rightarrow HS^- + 3\ H_2O + OH^-$	NA	−165

Notes: (a) standard conditions; (b) conditions prevailing in anaerobic ecosystems (Valdez-Vazquez et al., 2005).

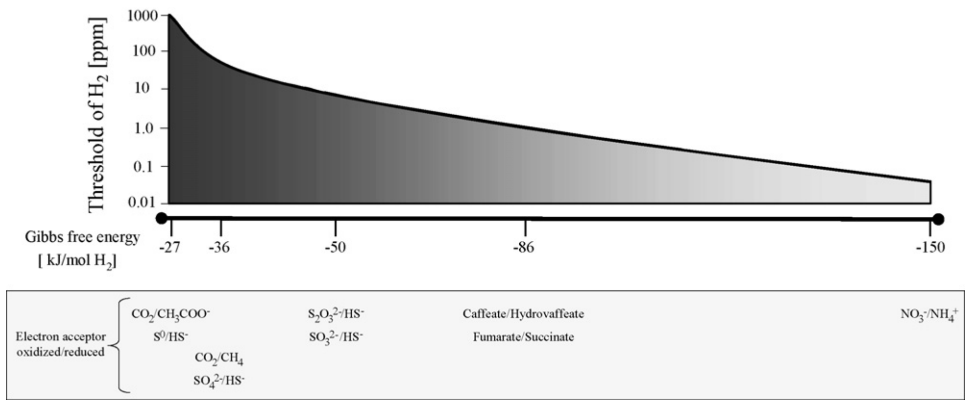


Fig. 2. Relationship between Gibbs free energy of electron acceptor on the threshold of H_2. Adapted from [12].

Therefore, according to Fig. 2, microorganisms that use nitrate as the electron acceptor are the most efficient in using H_2 at very low concentrations (0.02 ppm). In spite of this, NRB and/or SRB can only compete with methanogens and homoacetogens for the available H_2 when H_2 is the limiting resource and sulfate or nitrate are in excess [100]. For this reason, hydrogenotrophic methanogens are the main H_2-consuming microorganisms in most anaerobic environments [55,81,100].

3. Induction of H_2 accumulation in anaerobic consortia

In most anaerobic environments, the H_2 consumption is carried out very quickly by different microbial groups. Contrary to this natural fact, our interest is to propitiate the H_2 accumulation in order to use it as fuel. Therefore, H_2 accumulation is linked with the inhibition of H_2-consuming microorganisms such as hydrogenotrophic methanogens and autotrophic acetogens being the main ones when nitrate and sulphate are absent or negligible. Only a few reports have observed H_2 consumption by autotrophic acetogens during H_2 production from carbohydrate fermentation. However, when this phenomenon is presented, the growth limitation of autotrophic acetogens by means of CO_2 removal is possible [67]. On the other hand, methanogen inhibition is possible by means of a biokinetic control, heat-shock treatment and chemical compounds.

When non-sterile substrates are used, the proliferation of new non-inhibited methanogens is possible. Therefore, the inhibition method has to be continuous. In literature, the main methanogen inhibitors are chemical compounds such as 2-bromoethanesulfonate, acetylene, ethylene, ethane, methyl chloride, methyl fluoride and lumazine. Furthermore, recent work has used nitrate addition (1000 mg-KNO_3/L) for methanogenesis inhibition to enhace H_2 production [36]. In additional, other kinds of methanogen inhibitors have been utilized such as a biokinetic control which consists of applying environmental conditions at which methanogens cannot grow, i.e. low pH, large dilution rates causing the complete wash-out of methanogens, heat-shock pretreatment at ~100 °C during several minutes causing the total destruction of methanogens among other microorganisms. It can be observed that there are many options for methanogenesis inhibition; selection of an inhibition method that will depend on investment and operational costs, technical feability and complexity, inhibition effectiveness during the entire fermentation time, stabilization times of the inoculum, friendliness to H_2-producing microorganisms, compatibility with the H_2 metabolism, inocula

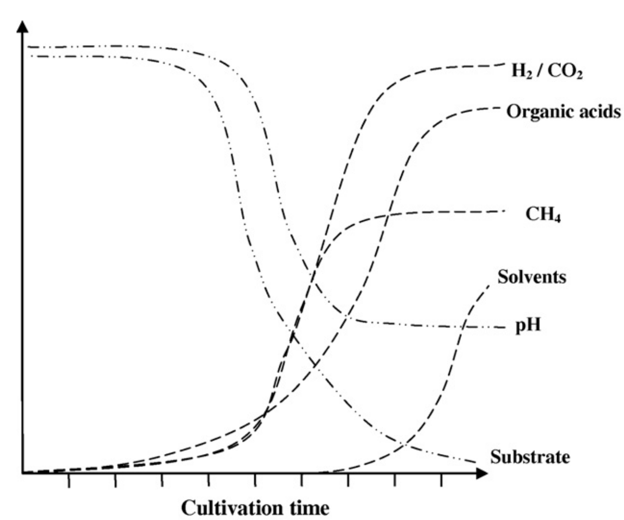


Fig. 3. Typical performance of batch hydrogen fermentation.

origin and substrate type, among others. Investigators have used one or more of these inhibition methods in studies of H_2 production by anaerobic consortia although it is not still clear which is the best. In this way, possibly the simplest and most economic method is the biokinetic control, mainly utilization of low pH.

3.1. Biokinetic control

In this review, we refer to biokinetic control as methanogen inhibition by acidogenesis due mainly to organic acid accumulation during fermentation. This is achieved by overloading the batch reactors with organic matter generating a fast and large organic acid accumulation which reduce pH to critical levels for methanogens. On the other hand, the growth rates could also be used to eliminate methanogens in continuous reactors with liquid substrates. This is possible because the growth rate of methanogens is lower than that of H_2-producing microorganisms. Therefore, methanogens are washed out of the reactor utilizing high dilution rates [16].

3.1.1. Acidogenic conditions

When pH is not controlled, a drop in pH due to organic acid accumulation is linked to H_2 evolution as is shown in Fig. 3. Thus, under certain low pH's, methane evolution stops and H_2/CO_2 are the main gases produced [70]. Most methanogens grow over a relatively narrow pH range (6–8). Some acidophilic species such as *Methanobacterium espanolae*, grow at pH's between 5.6 and 6.2 but are unable to grow and produce methane at pH 4.7 [23,84] showed that inhibition of methanogenesis activity is necessary for avoiding methane formation from evolved H_2 when an anaerobic inoculum is used. So low pH (around 5.0) is effective for inhibiting methanogenesis activity and obtaining an inoculum rich in H_2 producers. The acclimatization times reported to obtain a methanogen-free inoculum generating H_2 and organic acids are variable among different work and fall in a range between 3 and 30 days [84,8,50,92]. In order to enhance H_2 production, inoculum enrichment relevance at low pH was established by [8] where inoculum acid-enrichment during 80 h produced ~330 times more H_2 compared with the control (without enrichment).

The experimental observation that H_2 production is improved under acidogenic conditions were also confirmed by [99] in anaerobic reactors during shock loads. They proposed that this behavior could be corroborated by theoretical analysis of an equilibrium between formate and H_2 through a formate:hydrogen lyase:

$$H_{2g} + HCO_3^- \rightarrow HCOO^- + H_2O \quad \Delta G^{\circ\prime} = -1.3\,\mathrm{KJ/mol} \tag{8}$$

Based on chemical equilibrium, the following equation can be written as:

$$\frac{[HCOO^-]}{H_{2\,aq}} = K\,K_{CO_2}\;pCO_2\,K_A/K_{H_2}[H^+] \tag{9}$$

where K_{H_2} is the Henry's constant for H_2, K is the equilibrium constant (1.31 at 35 °C), K_{CO_2} is the Henry's constant for carbon dioxide and K_A is the first dissociation constant for carbonic acid. From Eq. (9), it can be seen that the ratio of formate to H_2 varies exponentially with pH. As a consequence, H_2 production is more probable to dominate under acidogenic conditions according to the following equation:

$$\frac{[HCOO^-]}{H_{2\,aq}} = \frac{K\,K_{CO_2}\,pCO_2\,K_A}{K_{H_2}} \times 10^{pH} \tag{10}$$

Recent studies have shown the effect of different pH's on biological H_2 production. For instance, the initial pH in batch test

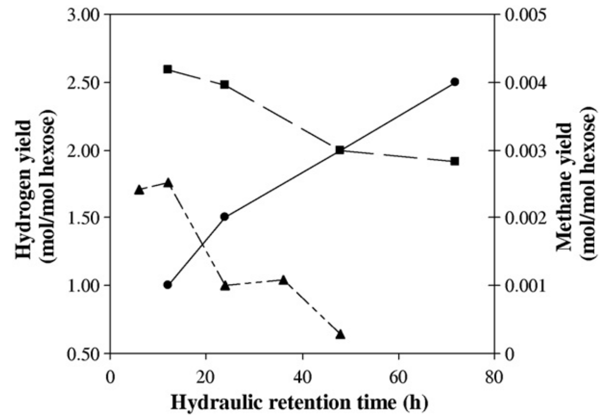


Fig. 4. Effect of high hydraulic retention times on hydrogen and methane yields. (■), (▲) hydrogen yields from two different works; (●) methane yield (adapted from data published by [85,7]).

using sucrose and starch as organic substrates was studied by [35]. They found that H_2 production was strongly affected by initial pH, since total H_2 production was initially higher at pH 4.5 than that at pH 6.5 for both sucrose and starch. Contrary to this, H_2 production rate and lag phase were negatively affected by an extremely low pH of 4.5. The above-mentioned behavior was because all batch fermentations reached a final pH near 5.0 due to rapid organic acid accumulation independently of an initial pH. Thus, H_2 producers probably could not adapt to the fast changes in the environmental conditions caused by the rapid depletion of pH which might have resulted in a metabolic alteration and subsequent inhibition of H_2 production, mainly those with initial pH 6.5. In general, the optimal pH for H_2 production is between 5.0 and 6.5 where a pH change of 0.5 units from the optimally determined pH decreased H_2 production efficiency by 20% [18,48,49,56,98]. Therefore, it is necessary to detemine the optimal acidic pH for each inoculum. In this way, the maintenance of the culture at pH 5.0–6.5 is crucial for methanogenesis inhibition and possibly other H_2-consuming microorganisms such as SRB and H_2-consuming acetogens [24,53,72,37,5,66]. In addition, maintenance of a moderately acidic pH creates good conditions for H_2 production from anaerobic cultures without causing a drastic change in environmental conditions for H_2 producers, although isolated reports have shown high H_2 production at a pH of 4.5 degrading 5.5 g-carbohydrates/L [19]. The role of low pH goes beyond being a control parameter during fermentation since it is linked to the shift of metabolic pathways and hydrogenase activity (see Section 6). The main limitations of this method are acclimatization time and the eventual presence of acidogen H_2-consuming microorganisms.

3.1.2. Short hydraulic retention times

Hydraulic retention time (HRT) is defined as the volume of the reactor/volumetric flow and is also known as the inverse of the dilution rate (D). The continuous stirred tank reactors (CSTR) or chemostat could be used to select microbial populations whose growth rates are able to catch up to the dilution caused by continuous volumetric flow. In this way, only microbial populations with growth rates larger than the dilution rate can remain in the reactor ($\mu_{max} > D$). Based on this, high dilution rates (short HRT's) could be used to cause the complete wash-out of methanogens since the specific growth rates of methanogens are much shorter than those of H_2-producing bacteria (0.0167 and 0.083 h^{-1}, respectively). Fig. 4 shows the typical effect of HRT's on hydrogen and methane yield in two different papers using a chemostat culture degrading soluble substrate [85,7]. It can be observed that in two independent studies, the highest H_2 yields were linked to short HRTs (or high D).

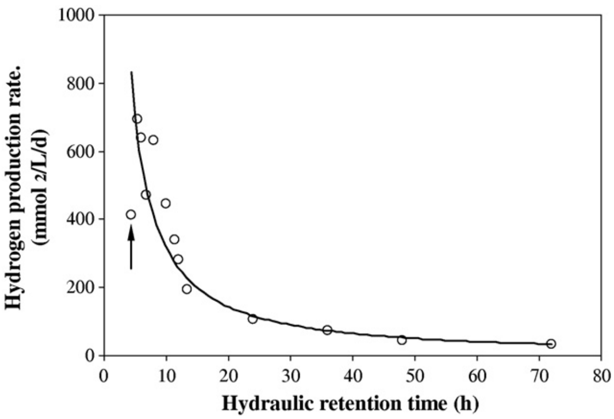


Fig. 5. Wide range of hydraulic retention time affecting hydrogen production rates. Arrow indicates probable wash-out of the reactor by extreme high hydraulic retention time (based on data reported by [85,47,7,30]).

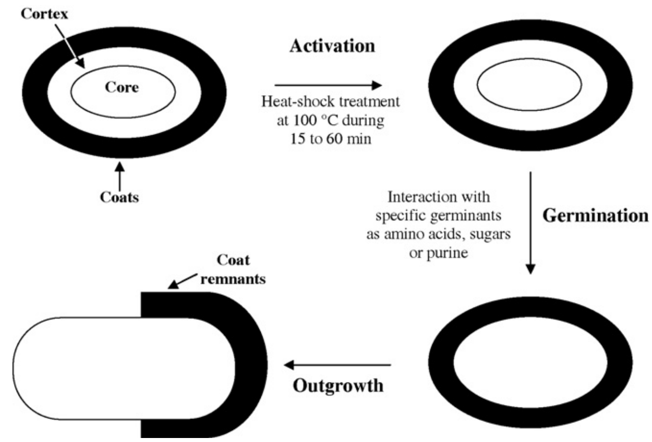


Fig. 6. The transition of dormant spores to active vegetative forms. Adapted from Ref. [75].

In opposition to this, methane yield was strongly affected by short HRT's. The H_2 fermentation pattern may shift to methanogenic fermentation if the HRT is increased. [7] calculated the maximum specific growth rate (μ_{max}) at which sewage sludge acclimated to produce H_2 finding a value of 0.172 h^{-1}. Therefore, based on the above-mentioned results, a dilution rate $<0.172\ h^{-1}$ (or HRT > 6 h) is recommended.

Fig. 5 shows the H_2 production rates in a wide range of HRT's. This figure was made with data from four different reports [85,47,7,30]. It is possible to observe that in spite of the differences among the works (such as substrate, type of inocula, incubation temperature), the tendency is greatly conserved: at high HRT's, the H_2 production rate is diminished with a critical value of HRT of 6 h. Other reports found dissimilar values of H_2 production rates but with the same tendency: maximum H_2 rates were registered at HRT between 14 and 17 h [39,50]. Therefore, the results obtained from these studies demonstrate that a decrease in HRT's can also achieve methanogen wash-out, although this method only can be applied when non-complex or soluble substrates are used.

3.2. Heat-shock treatment

Some microbial species such as *Bacillus* and *Clostridium* have the capacity to sporulate when environmental conditions become hostile such as heat shock, changes in nutrients status, presence of deleterious chemicals, among others [22]. The spores are metabolically dormant and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals [75]. In anaerobic environments, the main spore-forming microorganisms are several genera of acidogenic bacteria. This fact has been used by several authors to eliminate or kill non-spore-forming microorganisms, mainly methanogens, by means of a heat treatment with inoculum typically at ~100 °C for 15–120 min. This treatment simultaneously selects spores of acidogenic bacteria that will germinate, producing H_2 when conditions are again favorable for growth. Optimal conditions for obtaining high populations of H_2-producing microorganisms seem to be 80 °C/3 h at 3% TS although it has been reported that in increasing the initial TS, the optimum heat-treatment time also increase [41,98]. The transition of dormant spores to active vegetative forms can be divided into three phases: activation, germination and outgrowth (Fig. 6). Spore activation is usually achieved by heating spores in aqueous suspension [27]. In this way, it is possible to say that heat treatment (also called heat-shock pretreatment, HSP) is good to eliminate vegetative cells and to *activate* the present spores in the inoculum. Later, germination is initiated by interaction of the spore with specific germinants, the most frequent nutrient germinant being L-alanine. Finally, outgrowth leads to the formation of a new vegetative cell [22]. In the literature, there are a wide number of reports studying these three phases with species of *Bacillus* spores but in smaller proportion with H_2-producing bacteria such as *Clostridium* [71]. Reports that have studied the most favorable conditions for spore germination along with the H_2 production are scarce in spite of its significance for fermentative H_2 production processes.

The HSP effectiveness for H_2 production was demonstrated by [42] using a heat-treated anaerobic digested sludge during 15 min degrading the organic fraction of municipal solid waste (OFMSW) incubated at 37 °C. The H_2 yield obtained with this heat-treated anaerobic sludge was similar to those found with a H_2-producing bacteria of the genus *Clostridium* (140 and 180 mL H_2/g OFMSW, respectively). In both cases, 60% of hydrogen and trace elements of methane were found. Later on, [40] determined that clostridia was the predominant H_2-producing bacteria in the heat-treated sludge by means of metabolite profiling. They found that the activity of heat-shocked sludge was significantly inhibited when the initial cellulose concentration exceeded 25 g/L. Also, the sludge activity was influenced mainly by the ratio of the initial substrate concentration (S_0) to initial sludge density (X_0). Thus, high H_2 generation was obtained from cellulose at low S_0/X_0 ratio. [64] presented an interesting work where effectiveness of low pH and HSP for methanogen elimination were compared. They found that greater H_2 yields were found with heat-treated inocula (pH 6.2 or 7.5) than that at low pH (6.2) without HSP. These results were important to establish HSP's superiority over low pH. However, the pH's used were not those reported as the best for H_2 production (pH ~ 5.5). Therefore, the HSP superiority over low pH is only relevant at pH 6.2 and 7.5 since H_2 performance could change at pH 5.5.

However, HSP has been used by several authors during short operation periods. Increasing evidence shows that a stable H_2 production and methanogen repression is not possible for long-term continuous operation [36]. In this respect, [82] utilized an anaerobic sludge treated thermally at 100 °C for 15 min for H_2 production using sucrose as the substrate in two types of continuous reactors. The first reactor was equipped with an activation chamber which exposed a third of the biomass at 90 °C for 20 min, while the second reactor was not equipped with an activation chamber but the biomass was exposed to an initial heat-treatment. Their results indicated that the performance of the first reactor was better than that of the second reactor. Both initial and repeated heat treatments of the biomass during operation enhanced H_2 percentage. However, the highest H_2%

was obtained from the first reactor (51% vs. 43%). From these results, it seems to be that the initial HSP is not enough to maintain free methanogen reactors during the operation even when the pH was 5.5. Although repeated heat treatments seemed to be effective for eliminating methanogens during operation, it was not reasonable.

HSP has also been used for eliminating lactic acid bacteria (LAB) that is present in some waste such as that produced in bean curd manufacturing. LAB may cause the inhibition of H_2-producing bacteria by excretion of bacteriocins [63]. Bacteriocins are proteins with bactericidal activity directed against many Gram-positive bacteria, including *Clostridium*. These compounds are frequently found as secondary metabolites produced by diverse microorganisms such as LAB and genus *Bacillus* [28]. Several authors have reported that in decreasing the pH there was an increase in bacteriocin production which are more active at low pH [68]. From these observations, it can be pointed out that the heat treatment of organic waste could be effective for preventing H_2 production inhibition by LAB.

3.2.1. Nutritional and environmental requirements for germination

When HSP is used, spores of H_2-producing microorganisms are selected. The critical step to obtaining vegetative cell producers of H_2 from dormant spores is the germination step (Fig. 6). In consequence, it is necessary to provide conditions and nutriments required for a fast and optimal germination. Only a few authors have studied the effect of initial substrate and pH levels for germination of H_2-producing bacteria spores. In spite of using different heat-treated inocula, three different studies conclude in that the optimal pH for H_2 production is 5.5, where optimal substrate concentration was between 7.5 and 15 g COD/L in liquid cultures [94,95,17].

When all vegetative cells are killed, it is normal to find a lag time of several days for H_2 production to begin. This lag time will depend on several factors such as initial pH, nutrient concentrations, temperature, germinant availability, among others. Typical lag times for H_2 production under mesophilic conditions are 2–4 days. However, [90] observed a much bigger lag time under thermophilic conditions (>4 days), utilizing heat-treated micloflora fermenting solid organic sustrate. To this respect, [89] found that the addition of specific nutrients (termed germinants such as aminoacids) was necessary for optimal and complete germination at thermophilic incubation obtaining lag times <1 day for H_2 production. Thus, in absence of germinants, the best temperature for spore germination seems to be at 37 °C.

It is possible to say that HSP is effective and fast to obtain inocula for H_2 production although it is necessary that further studies on nutritional and environmental requirements for optimal germination of selected spores achieving a large H_2 production be conducted. On the other hand, this method does not avoid the proliferation of H_2-consuming microorganisms coming from non-sterile feedstock which could cause H_2 production depletion [82]. It is then necessary to apply HSP whenever the H_2 production decreases, causing an increase in production costs.

3.3. Chemical inhibitors

In literature, there have been reports of many chemical substances that inhibit methane formation for methanogenic archaea that have different specificities and act at different concentrations. These compounds have been applied to study the importance of methanogenesis in the environment and can be classified into two major groups: nonspecific and specific inhibitors.

Chloroform, fluoroacetate and acetylene are some examples of "nonspecific" inhibitors for methanogens. Chloroform ($CHCl_3$) is known to block the function of corrinoid enzymes and to inhibit methyl-coenzyme M reductase [65]. $CHCl_3$ not only inhibits methanogenesis, but also inhibits partially acetate-dependent sulfate reduction and possibly H_2-dependent homoacetogenesis. On the other hand, fluoroacetate (FCH_2COO^-) has extensively been used to block acetate metabolism since it is converted to fluorocitrate which then inhibits the activity of aconitase in the tricarboxylic cycle [44]. Fluoroacetate also inhibits acetoclastic methanogenesis. Some studies indicate that fluoroacetate is possibly activated as acetate before it exerts its adverse effect [11]. Methanogenesis has been inhibited by acetylene (C_2H_2) in anaerobic sediments, anaerobic paddy soils and rumen fluid. [80] found that the intracellular ATP content of all of the methanogens dropped dramatically after exposure to C_2H_2. Moreover, cells of *Methanospirillum hungatei* and *Methanobacterium bryantii* exposed to C_2H_2 lost their ability to maintain a transmembrane pH gradient. Sprott and collaborators suggested that exposure to C_2H_2 resulted in a decline in methanogenic functions which require a H^+-flux, including ATP synthesis, Ni^{2+} uptake and methanogenesis.

Chloroform and fluoroacetate use for H_2 production can be an environmental contradiction since these inhibitors have halogen molecules and their application is directly on the solid/liquid substrate which would cause problems for their disposal. Yet, acetylene is a gas which can be utilized in the headspace for H_2 production without disposability problems.

Other chemicals like 2-bromoethanesulfonate (BES) and 2,4-pteridinedione (lumazine) are an example of "specific" inhibitors [78]. BES ($BrCH_2CH_2SO_3^-$) is a structural analog of coenzyme M (2-mercaptoethanesulfonic acid), the methyl carrier in the final reductive step of methanogenesis. The coenzyme M seems to be unique to methanogens but not in *Bacteria* or *Archaea* [74]. Coenzyme M accepts methyl groups generated from methanol or CO_2 to form methylcoenzyme M that was subsequently demethylated by H_2 or reduced cofactors to generate methane and regenerate Coenzyme M [77].

The pterin lumazine compound is a structural analog of some cofactors in methanogenesis like methanopterin and reduced forms deazaflavin F_{420} [14]. This pterin inhibits methanogenesis from H_2 and CO_2 or from H_2 and methanol. In contrast, growth of non-methanogenic archaea, numerous eubacteria and eukaryotes are not strongly affected. The *in vitro* results, together with *in vivo* observations, suggest that steps described below are potential sites of action for lumazine: (1) CH_3-SCoM reduction to methane by the HS-HTP-dependent CH_3-S-CoM methylreductase, and (2) H_2-dependent HS-CoM and HS-HTP regeneration by the CoM-SS-HTP disulfide reductase system. An attractive characteristic of lumazine is that spontaneous resistance has not been observed, implying a resistance frequency at least five orders of magnitude below that seen with BES [59].

BES has been used in control of methanogenesis inhibition in H_2 production studies, while lumazine has not. Sparling et al. [79] compared the effectiveness of air, BES and acetylene as methanogenic inhibitors. At 1% (v/v) in the headspace, acetylene was as effective as BES in inhibiting methanogenic activity in batch anaerobic composters with an undefined cellulolytic consortium derived from anaerobic digesters. Acetylene also had no effects on the rate and amount of H_2 produced from a pure of *Clostridium thermocellum* grown under the same conditions. Valdez-Vazquez et al. [93] also used acetylene to inhibit methanogenic activity during the batch fermentation of paper wastes. In this study, acetylene was also superior to the BES regarding H_2 production.

One of the main advantages is that acetylene is a cheap gas that will exit the bioreactor with the H_2-rich gas stream. Moreover, acetylene has a lower cost than BES. It does not accumulate in solid materials and does not interfere with hydrogenase activity [80,32]. In addition, the inocula do not require acclimatization time for H_2

production when acetylene is used. In this way, acetylene can be an excellent candidate for large-scale industrial production.

4. Structure, diversity and dynamics of H_2-producers communities

Hydrogen production using anaerobic consortia provides many advantages, the main one being that organic waste or wastewater could be used without sterilization. This may confer large economic profits to the process. In order to enhance the process performance and maintain an attractive H_2 production, it is advisable to gain insight on the community structure and dynamics. For years, culture-based studies were carried out to maintain and evaluate process conditions. However, those techniques have large limitations when microbial communities are studied. Moreover, these cultivation methods are time-consuming, labor-intensive and susceptible to bias toward non-predominant culturable microorganisms [3]. Recent studies of biological H_2 production have used indirect methods (metabolite distribution, enrichment methods and microscope examination, etc.) and molecular biological techniques (DGGE, DNA-cloning analysis, dot-blot hybridization, terminal restriction fragment length polymorphism) in order to determine microbial composition in hydrogenogenic processes.

The metabolite distribution has been monitored along with the characterization of microbial populations. Regarding this, the metabolites most commonly formed during H_2 fermentation are acetate, propionate, butyrate, ethanol and butanol. In this way, several authors have suggested that *Clostridium* species are dominantly present, in H_2 producer systems [42,39,40]. In additional, another indirect approach to determining the microbial structure is the method used for inocula enrichment with H_2-producers. In this way, both heat treatment and acidogenic operation enriched with anaerobic spore-forming bacteria related with the *Clostridium/Bacillus* groups. This suggestion was corroborated by [50] who used scanning electron microscopy (SEM) to observe the microstructure of the H_2-producing acidogenic granules. The SEM images illustrated that granules were typically composed of the spore-forming, rod-shaped bacteria and fusiform bacilli. The characteristic granules suggested that the dominant species might be *Clostridium* sp.

Those studies, however, do not offer a precise estimation of the diversity, composition and dynamic of microbial populations or their affectation by different operational parameters. Recent advanced molecular techniques have been developed to analyze the structure and species composition of microbial populations. For instance, denaturing gradient gel electrophoresis (DGGE) separate PCR-amplified 16S ribosomal DNA fragments in polyacylamide gels containing a linearly increasing gradient of denaturants [58]. In this way, [51] studied the start-up of two acidogenic reactors under meso and thermophilic conditions with methanogenic granular sludge as inoculum fermenting dairy wastewater. They monitored the microbial community dynamics by DGGE over a 71-day period quantitatively monitored by using dot-blot hybridization with rRNA-targeted oligonucleotide probes. The authors found that a pH drop to 5.5 caused DGGE community fingerprints for bacteria and archaea populations to shift at 13 days of operation. This observation was accompanied by a decrease in methane formation and an increase in H_2 and volatile organic acid production. Dot-blot hybridization indicated that the bacterial population in the mesophilic acidogenic reactor increased from 63.1 to 90.3%, while archaea population significantly decreased from 34.1 to 4.3%. On the other hand, DGGE fingerprints indicated that a bacterial community shift in the thermophilic sludge was more significant than in the mesophilic sludge within the first 13 days. That study revealed that less than 2 weeks were needed to establish a desirable microbial population in the acidogenic reactors but a longer period, up to 71 days, was necessary to obtain a microbial community showing stable metabolic activity. In that work, pH and temperature were major factors in the microbial community shift during the start-up of the acidogenic reactors.

In another work, Fang et al. [20] studied the change in the microbial community due to the change in pH by DGGE profiles. In this way, the number of bands increased with pH due to the presence of methanogens as evidenced by the increased methane production. This work confirmed again that pH is a factor that limits the growth of methanogens and causes growth of predominanly H_2-producing microorganisms. In order to know the microorganisms that were in great proportion and were responsible for the H_2 production, they made the phylogenetic analysis on the ribosomal DNA sequences. Thus, three *Clostridium* species were found to be predominant (64.6%) and the rest were affiliated with Enterobacteriaceae (18.8%), *Streptococcus bovis* (3.1%). The remaining affiliations were not identified.

On the other hand, Ueno et al. [86] found that the product distribution and bacterial community were highly dependent on the dilution rate in a thermophilic chemostat reactor fermenting glucose and cellulose by methanogenic microflora. Thus, methane production was at a maximum at 0.67 and 0.33 day^{-1} on glucose (66.8 mmol/L day) and cellulose (30.6 mmol/L day), respectively, without significant metabolite accumulation. In contrast, H_2 production was at a maximum at 4.81 day^{-1} on glucose (24.7 mmol/L day) and cellulose (42.3 mmol/L day). The predominant metabolites were lactate on glucose and acetate/ethanol on cellulose. That was probably due to lower growth of methanogens comparated to acidogens. This difference in growth rates might have resulted in a wash-out of methanogens from the reactor. Thus, low dilution rates and different DGGE profiles showed that the microbial population was very similar during methane fermentation. However, when the dilution rate was increased DGGE profiles indicated very different changes on glucose and cellulose. Thus, the DGGE profile on glucose at 2.67 and 4.181 day^{-1} consisted of several major bands related with *Bacillus* genus. In opposition to this, the DGGE profile on cellulose at 4.81 day^{-1} showed that two bands were predominantly related to *Clostridium* genera. For that reason, the investigators suggested that a difference in H_2 yield could be caused by different populations of microorganisms in each microflora.

Sung et al. [82] used terminal restriction fragment length polymorphism (T-RFLP) to identify H_2-producers mixed communities in mesophilic continuous flow reactors using sucrose and heat-treated inocula. The reactor was equipped with an activation chamber that exposed a fraction of the settled sludge to a temperature of 90 °C from 20 min. Their results indicated that two major groups of *Clostridium* species were dominant during the first 15 days of operation. The first dominant *Clostridium* group was composed of the following species: *C. beijerinckii*, *C. botulinum, C. putrificum* and *C. sporogenes*. The second dominant population was identified to be like *C. butyricum*. The authors found that a decrease in H_2 production was accompanied with a decrease in the total of *Clostridium* species and *vice versa* in the first 15 days of operation. However after day 18, the two *Clostridium* species groups ceased to grow and *Bacillus* species became dominant after day 22. They concluded that it was necessary to apply repeated heat treatments in order to maintain H_2 production in a continuous flow reactor.

In 2001, Ueno and collaborators reported two crucial studies that demonstrated that microbial communities could achieve H_2 yields similar to those achieved by pure cultures. They studied H_2-producers in microbial communities through isolation of the microorganisms by both plating and DGGE of the PCR-amplified V3 region of 16s ribosomal DNA. *Thermoanaerobacterium thermo-*

saccharolyticum was isolated in the enrichment culture and was detected with strong intensity by DGGE. Two other thermophilic cellulolytic microorganisms, *Clostridium thermocellum* and *Clostridium cellulose*, were also detected by DGGE although they could not be isolated. *T. thermosaccharolyticum* grown in pure culture demonstrated a high H_2 yield of 2.4 mol/mol-glucose. In this case, the fermentation pattern was similar to that observed for the H_2 fermentation of wastewater by the microflora [87,88]. This result suggested it was possible to utilize anaerobic consortia with high H_2 production performances.

Recent development and application of molecular biological techniques have confirmed the observations done by indirect methods on H_2-producer populations. Moreover, these techniques have provided an additional and valuable tool for studying the diversity, composition and dynamic of microbial communities.

An analysis of the presented work allows one to observe that there is an association between high H_2 yields and low microbial diversity of communities. Also pH, temperature, dilution rate and substrate utilized for H_2 production have helped to establish specialized anaerobic consortia. In general, when low pH, thermophilic temperature, high dilution rate and substrates difficult to degrade are used in biological H_2 production, the microbial diversity is low but maximum H_2 yields are obtained (already 2.0 mol/mol glucose). In these specialized anaerobic consortia *Clostridium* sp. is the predominant genera. The main identified species are: *C. thermocellum*, *C. cellulose*, *C. thermosuccinogenes*, *C. beijerinckii*, *C. botulinum, C. putrificum* and *C. sporogenes*. Others genera found are *Thermoanaerobacteroids proteolyticus* and *Thermoanaerobacterium thermosaccharolyticum* that produce H_2 and carbon dioxide with production of acetate and ethanol. The *Clostridium* genus is moderately spread in H_2 producers systems. Even in a phototrophic system producing H_2 from acidified wastewater, it was found that 19% of species were formed by clostridia [103].

In addition to clostridia, there are other H_2-producer genera such as facultative anaerobes like *Enterobacter* sp. These genera are able to produce H_2 and organic acids as by-products from organic substrates. Contrary to clostridia, those are not sensitive to oxygen and their H_2-producing activities are not totally inhibited by the presence of oxygen in a feeding medium. In spite of this, H_2 yield from the *Clostridium* species is generally higher than that from *Enterobacter* species: 2 and 1 mol/mol hexose, respectively [31]. For this reason, it is preferable to enrich the anaerobic inocula with *Clostridium* species, as previosly discussed (archive similar H_2 yield to pure cultures). Nevertheless, it is necessary to provide the optima conditions (pH, temperature, substrate principally) and diverse specific germinants (such as amino acids, mineral salts) for successful spore germination due to the enrichment with *Clostridium* spores. This may increase the process costs. However, these requirements are not as demanding as with pure cultures.

5. Biochemical pathways for H_2 production

Fig. 7 shows the biochemical pathways utilized by clostridia for the conversion of carbohydrates to H_2, CO_2, organic acids and solvents. These biochemical pathways are similar in diverse Clostridium species, i.e., *Clostridium acetobulylicum* and *Clostridium thermocellum* except that the pathway for production of acetone and butyric are absent in *C. thermocellum*. According to literature, two main phases can be distinguished during the batch

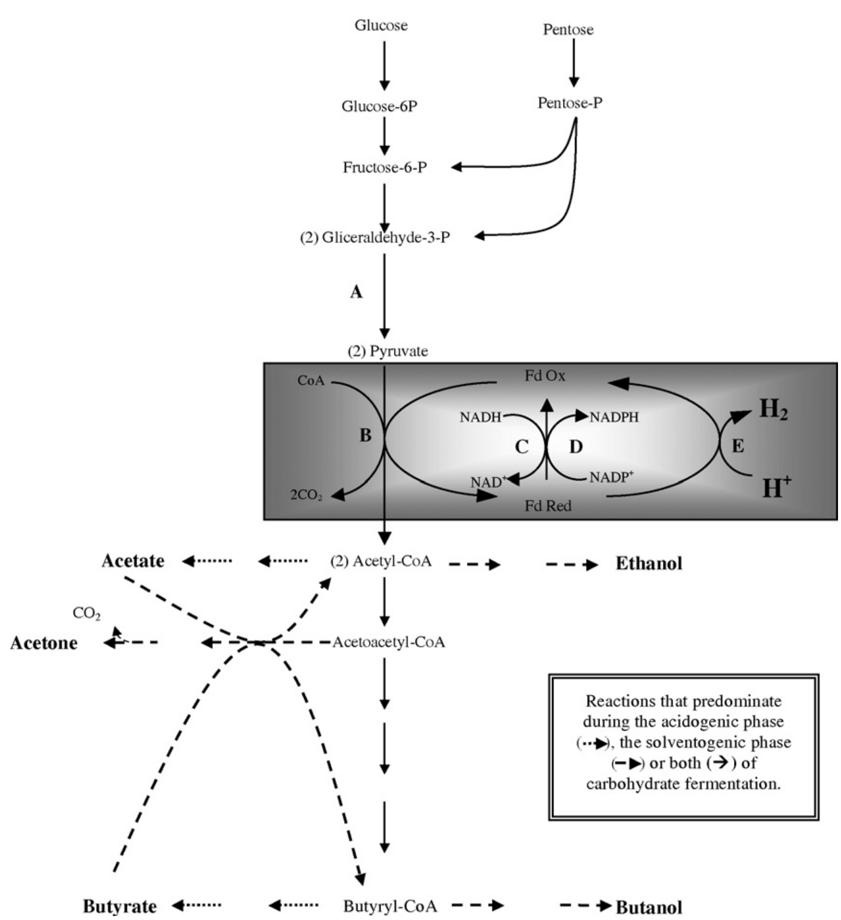


Fig. 7. Biochemical pathways utilized by Clostridia genera for the conversion of carbohydrates to hydrogen, carbon dioxide, organic acids and solvents. Adapted from Ref. [34].

fermentation: the acid production phase (Fig. 7) and the solvent production phase (Fig. 7).

Firstly, during the via Embden-Meyerhof pathway, 1 mol of hexose is metabolized to 2 mol of pyruvate with the production of 2 moles of reduced nicotinamide adenine dinucleotide (NADH) and 2 mol of adenosine triphosphate (ATP). Clostridia can also utilize the pentose phosphate pathway for the conversion of 3 mol of pentose to 5 mol of ATP and 5 mol of NADH [73]. Pentose sugars are fermented to pentose 5-phosphate, Then, by means of the transketolase-transalsolase sequence, fructose 6-phosphate and glyceraldehyde 3-phosphate are produced and can enter to glycolytic pathway. Later, pyruvate generated from fermented hexose/pentose sugars is cleaved by pyruvate ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to generated acetyl-CoA, reduced ferredoxin and carbon dioxide. The acetyl-CoA produced is the essential intermediate in both acid-producing and solvent-producing pathways.

Acetyl-CoA can be phosphorylated by the phosphotransacetylase-kinase or phosphotransbutylase-kinase system to generate acetate or butyrate and ATP. When organic acids are generated, there are not any reductions and reduced ferredoxin is able to transfer electrons to a hydrogenase that permits the use of protons as a final electron acceptor. Thus, ferredoxin is re-oxidized and molecular H_2 is released from the cell. The proton reduction (H_2 evolution) is essential in pyruvate fermentation or in the disposal of excess electrons.

Under certain conditions (e.g. high H_2 partial pressure), the law of mass action limits the formation of H_2 and the cell is forced to channel electrons through NADH:ferredoxin oxidoreductase to reduce acetyl-CoA and butyryl-CoA to ethanol and butanol, respectively [15]. Thus, solvent production involves a switch in the carbon flow from the acid production pathway to the solvent production pathway (see Fig. 7). These reactions require two sets of dehydrogenases to achieve the necessary reductions to produce ethanol and butanol. Since solvent production involves reductions, ferredoxin is unable to transfer electrons to a hydrogenase for H_2 evolution. For this reason, it is necessary to avoid the conditions that force the cell to switch the acidogenic to solventogenic fermentation.

6. Hydrogenases

The enzymes directly involved in the metabolism of molecular H_2 are named hydrogenases and carry on the reversible oxidation of molecular H_2:

$$H_2 \leftrightarrow 2H^+ + 2e^- \tag{11}$$

Hydrogen oxidation is coupled to the reduction of electron acceptors such as oxygen, nitrate, sulphate, carbon dioxide and fumarate, whereas proton reduction (H_2 evolution) is essential in pyruvate fermentation or in the disposal of excess electrons. Many microorganisms have hydrogenases and some of these enzymes have been found to contain metal atoms in the active site. Hydrogenases can be classified into three groups with respect to the metals present in the active sites: the "only" [Fe] hydrogenases, the [NiFe] hydrogenases and the [NiSeFe] hydrogenases. These enzymes present important differences in terms of specific activity and bidirectionality [21].

Two families of Fe-Hydrogenases are recognized in particular. One of them can be found in strict anaerobic strains like *Clostridium pasteurianum* [2]. This genus has two divergent hydrogenases: the bidirectional hydrogenase (*Cp*I) and the H_2-oxidizing (uptake) hydrogenase (*Cp*II) [9,25]. Therefore, this work will focus on bidirectional *Cp*I.

6.1. Structure of Fe-Hase I from C. pasteurianum

3D structure of Fe-Hydrogenase I from *C. pasteurianum* is known [69]. This enzyme consists of a binuclear iron site bound to a (4Fe-4S) cluster by a bridging cysteine. The structure of this protein raises four cysteine ligands of the active site, some residues lining the active site cavity and also amino acids that could be in

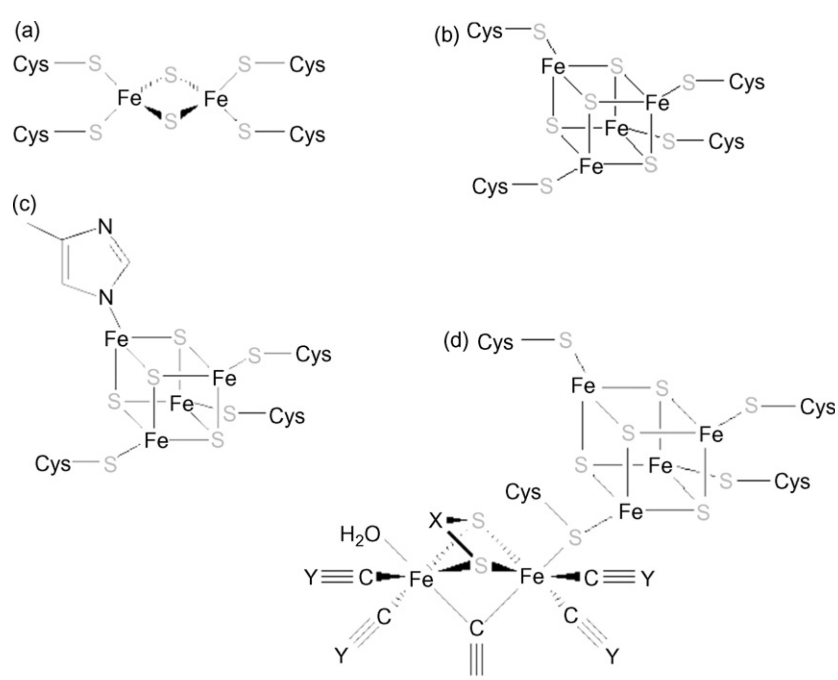


Fig. 8. Structure of iron-sulphur clusters from *Cp*I of *Clostridium pasteurianum*. (a) irons tetrahedrally coordinated from [Fe_2S_2] cluster in N-terminal FS2 domain; 9b) two [Fe_4S_4] clusters from FS4A-FS4B domain which is adjacent to the active site domain; (c) single [Fe_4S_4] cluster from FS4C domain which is positioned between the FS2 and FS4A-FS4B domains and (d) two subclusters [Fe_4S_4] and [Fe_2] (named H-cluster) from the active site domain. Adapted from Ref. [33].

the H_2 and H^+ channels connecting the hidden active site to the exterior [97]. In general, the *Cp*I structure consists of four domains: three small domains and one large active site domain [69,62]. The small domains connect four iron-sulphur clusters and are known as FS4A-FS4B, FS4C and FS2.

The N-terminal FS2 domain binds a $[Fe_2S_2]$ cluster where iron atoms are tetrahedrally coordinated both by two inorganic sulphurs and by sulphurs provided by four conserved Cys residues (Cys33, Cys46, Cys49 and Cys62 in *C. pasteurianum*). This cluster type was originally found in chloroplast membranes and is known as a chloroplast type or plant type cluster (Fig. 8a).

The FS4A-FS4B domain is adjacent to the active site domain and contains two $[Fe_4S_4]$ clusters. Each $[Fe_4S_4]$ cluster is coordinated both by four inorganic sulphurs and by sulphurs provided by four conserved Cys residues (Cys157, Cys190, Cys193 and Cys196 for FS4A and Cys147, Cys150, Cys153 for FS4B). This cluster was originally found in bacteria and is termed bacterialtype 2× $[Fe_4S_4]$ ferredoxin (Fig. 8b).

The FS4C domain is positioned between the FS2 and FS4A-FS4B domains and consists of two α-helices linked by a loop that binds a single $[Fe_4S_4]$ cluster by one His and three Cys residues (His94, Cys98, Cys101 and Cys107, see Fig. 8c).

The active site domain of the Fe-Hases contains an Fe-S centre termed the H-cluster [2] that consists of two subclusters, $[Fe_4S_4]$ and $[Fe_2]$, which are connected by one Cys thiolate. The $[Fe_4S_4]$ subcluster is coordinated by only three Cys (Cys300, Cys355 and Cys499) while the two iron atmos from $[Fe_2]$ subcluster has been termed Fe1 and Fe2 (proximal and distal with respect to the $[Fe_4S_4]$ subcluster, respectively). This di-iron subcluster is not coordinated by protein ligands. In *Cp*I, both iron atoms are octahedrally coordinated to five CO/CN ligands, three S ligands and one water molecule. In turn, F1 and F2 are linked by two S atmos and one CO or CN ligand (Fig. 8d).

6.2. Hydrogenase activity

It is known that environmental factors could affect the activity of the enzyme in the H_2 production process. Thus, studies in the 1980s found that factors such as pH and temperature have a direct influence on the activity of the enzyme.

6.2.1. pH

Several reports observed that the H_2 production initiation was carried out only after pH decreased to ~5.5. Studies found that the hydrogenase activity measured in whole cells from acid-producing cultures maintained at pH 5.8 was about 2.2 times higher than that measured in cultures maintained at pH 4.5 [4]. In general, hydrogenase activity (uptake and evolution) is low in cells maintained at a pH < 5.2 [26]. Also, [1] found that the activity of hydrogenase I increased steadily with decreasing pH with an optimum pH of 6.3. Thus, these studies on hydrogenase activity are directly correlated with those of H_2 fermentation showing that pH plays multiple roles in H_2 production.

6.2.2. Temperature

Adams and Mortenson [1] determined the effect of temperature on the rate of H_2 catalysis by hydrogenases I and II from mesophilic *C. pasteurianum*. The authors obtained the Arrhenius plot for calculating the activation energy (E_A) values and optimum temperatures for the reaction. With both enzymes in both assay systems, the plots were linear in the range of 15–50 °C. The rate of reaction decreased between 50 and 70 °C.

These *in vitro* results, together with the *in vivo* observations, suggest that the optimum temperature is approximately 50 °C. In different systems (reactors using soluble and solid substrates), it was observed that the specific H_2 production rate and the H_2 percentage increased with temperature. In both cases, an optimum H_2 production rate was achieved at 55 °C and the maximum percentage of H_2 was >60%. [102,76,92].

6.2.3. Iron concentration

Iron concentration seems to have an effect on hydrogenase activity since this enzyme consists of a binuclear iron site bound to a (4Fe-4S) cluster (see Section 6.1).

Lee et al. [43] studied the effect of the Fe concentration in the external environment on the H_2 production using sucrose solution and the mixed microorganism from a soybean-meal silo. The maximum specific H_2 production rate was found to be 24 mL/g VSS.h at 4000 mg $FeCl_2$/L (1760 mg Fe^{2+}/L). On the other hand, [41] found a much smaller optimal Fe^{2+} concentration (132 mg-Fe^{2+}/L) for H_2-producing composts using solid food wastes as a substrate. In spite of the unequal results, the authors agree that iron limitation could limit the hydrogenase activity along with H_2 evolution.

7. Inhibition of H_2 generation by products

The *Clostridium* species have two different metabolic pathways for H_2 production from carbohydrate fermentation: acidogenesis, which produces mainly organic acids like acetate and butyrate, and solventogenesis which generates solvents such as acetone and ethanol. Thus, when environmental conditions are favorable, *Clostridium* is able of modify their metabolism to any of these pathways. However, only the carbohydrate fermentation during acidogenesis generates high H_2 yields [73,34]. In this section, we will analyze the environmental conditions that allow the shift to solventogenic fermentation. These conditions have been studied better in the processes of solvents production like acetone-butanol.

7.1. pH and end products

The influence of pH has been recognized as a key factor in determining the outcome of H_2 fermentation (see Section 3). The pH is related with three important facts: (a) methanogen growth limitation; (b) H_2 production performance and (c) regulation of shift to solventogenesis. Fig. 3 shows that along with H_2 production, generated organic acids cause a drop in pH. If pH is not controlled, solvents could be generated at the end of fermentation. Thus, a pH decrease (by acids accumulation), typically to 4.5, could be used to induce the shift to solventogenesis along with H_2 production decline [98]. Although a decrease in pH is important for solvent production, pH itself is not the trigger [34].

With respect this, [10], studied the use of peptone and found that it avoided the abrupt pH drops in the system and allowed for further exploration of organic acids and pH effects on H_2 fermentation. The results suggested that the H_2 fermentation using the protein-containing substances as a substrate was beneficial in maintaining pH. As long as the pH was maintained at around 6–8, system inhibition was minimized. This strategy could be used to keep pH between 5.5 and 6.5 (optimal for H_2 fermentation), avoiding the solventogenic phase.

Along with pH, high organic acid concentrations could result in effects detrimental to H_2 fermentation. These reduced organic acids produced as end products of metabolism are toxic to the cell. Undissociated acids act as uncouplers which allow protons to enter the cell from the culture. Sufficiently high concentrations of undissociated acids could generate a collpase in the pH gradient across the membrane. Thus, the shift to solventogenesis has been related to a detoxification mechanism of the cell to avoid the inhibitory effects [34]. Organic acid concentration at which

solventogenesis could shift to differs among species although the concentration threshold was observed to be between 0.3 and 50 mM [29,91]. According to the Henderson–Hasselbalch equation, the undissociated acid concentration is pH-dependent being higher at a pH < pK_a. Thus, the pH theoretically has to be >4.8 (pK_a of butyrate) to avoid inhibition by high undissociated acid concentrations.

7.2. Partial pressure of hydrogen

The increase in the partial pressure of H_2 in the headspace during the fermentation has been associated with a decrease in H_2 production. [15] observed that when the H_2 partial pressure increases to a certain level in the reactor headspace, the culture will switch to alcohol production and produce much less H_2. Under these conditions, which resulted in a high concentration of H_2, the H^+/H_2 redox potential is lowered and the flow of electrons from reduced ferredoxin to molecular H_2 via the hydrogenase system is inhibited (see Fig. 7). The electron flow would be shifted to the generation of NADPH via the action of the appropriate ferredoxin oxidoreductase, resulting in an increase in the production of butanol and ethanol [34].

Several strategies have been developed to avoid the negative effect by the H_2 accumulation in the gaseous space. For instance, it has been reported that the removal of the rich gaseous phase in H_2 is cause by an inert gas. Another one is the *in situ* removal of H_2 from biogas by means of membranes. Below, we present representative investigations dealing with these two strategies and a discussion of their advantages/disadvantages.

7.2.1. Sparging with inert gas

The effects of H_2 on the metabolism and the fermentative pattern of anaerobic bacteria have been demonstrated by [54]. A H_2-producing mixed culture produces more H_2 when it is removed by nitrogen gas. A H_2 yield of 0.85 mol/mol consumed glucose was obtained after 5 HRT with the gas produced being 53.4% H_2. With nitrogen sparging at a flow rate approximately 15 times the H_2 production rate, the H_2 yield was 1.43 mol/mol consumed glucose. However, this method has a disadvantage in that a recirculation gas implies strong dilution with an excess amount of stripping gas to a low mole fraction. Thus its application at an industrial scale is not economically feasible.

Logan et al. [52] examined the biological production of H_2 with two techniques: an intermittent pressure release method (Owen method) and a continuous gas release method using a bubble measurement device (respirometric method). Under otherwise identical conditions, the respirometric method resulted in the production of 43% more H_2 gas from glucose than the Owen method. In the respirometric method, total pressure in the headspace never exceeded ambient pressure and H_2 typically composed as much as 62% of the headspace gas. This procedure only seemed to be adequate when from initial stages of the fermentation, the H_2 concentration was elevated. In opposition to this, the H_2 concentration increased with time in most of the fermentations. This is the reason why it is recommendable to concentrate the biogas until suitable levels to recover it later. Otherwise, a biogas can be obtained with a very variable H_2 composition.

Other investigators that observed this phenomenon were [92]. In this work, they studied the H_2 production from paper mill wastes using microbial consortia of solid substrate anaerobic digesters inhibited with BES or acetylene. In the second phase of tests, the headspace of the batch reactors was flushed with nitrogen after the first plateau of H_2 was reached, and subsequently incubated, with no further addition of inhibitor or substrate or inoculum. It was found that H_2 production resumed and reached a second plateau somewhat lower than the first one. This procedure was repeated a third time and an additional amount of H_2 was obtained. This procedure was named as intermittently vented solid substrate for anaerobic H_2 generation (IV-SSAH) and demostrates that inhibition by high partial pressure of H_2 is reversible. Thus, the total cumulative H_2 harvested in the three-cycle incubation was nearly double of that in the first cycle alone. Also, IV-SSAH procedure allowed to find that the partial pressure of H_2 at which H_2 production by anaerobic consortia was reversibly inhibited was 0.54 atm [91]. However, organic acids accumulation was responsible for a shift from acidogenesis to solventogenesis which reduced or stopped the H_2 production in subsequent production cycles (third to fourth cycles) in that study.

7.2.2. Membranes

[46] investigated the behavior and effectiveness of a silicone rubber membrane to separate biogas from the culture medium and the way in which the H_2 production of the fermentor was enhanced. The permeabilities of silicone rubber used were 4.58×10^{-8} cm^2/s kPa (35 °C) for H_2 and 2.60×10^{-7}cm^2/s kPa (35 °C) for CO_2. Reducing the partial pressure of biogas in the liquid increased H_2 production. H_2 gas yield was 5.14 mmol H_2/g glucose for the fermentor fitted with the silicone rubber membrane and 4.68 mmol H_2/g glucose for the fermentor without it. Nevertheless, the increase in H_2 production was only 9%. The authors argued that if the reactor were installed with more hollow fibers to obtain a greater membrane area, it could remove more H_2 gas immediately and the reactor would have a low H_2 partial pressure. These results did not surpass those found by sparging with an inert gas. In addition, the membrane installation in the interior of the reactor could have operational problems.

Up to this point, sparging with an inert gas seems to be the best option to reduce the H_2 partial pressure of those displayed in this subsection to increase the yields, rate and gross productions of H_2. However, more investigation is necessary on this topic to recognize that the option must be applied to large-scale industrial production.

8. Immobilized-cell systems

Immobilized-cell systems have become a common alternative to suspended-cell systems in continuous operation since they are more efficient in solid/liquid separation and can be operated at high dilution rates (or short retention times) without encountering washout of cells. Several studies found that immobilized-cell systems were suitable for continuous H_2 fermentation with pure cultures using a variety of natural and synthetic support matrices. However, information regarding utilization of immobilized mixed culture (such as sewage sludge) processes for H_2 production is still scarce [6].

Support for immobilized systems has to perform very specific characteristics for optimum performance. For intance, in biotreatment systems (i.e. biofiltration), the support must fulfill certain characteristics: (i) high surface area, for optimum microbial development, (ii) low bulk density for the easiest and cheapest carrier operation and (iii) high void fraction to the limit pressure drop and clogging problems, (iv) low cost [61]. In this way, a few investigations have been dedicated to search for supports that fulfill these characteristics to improve the yield of H_2 production. Studies focusing on these mixed cultures are shown below.

The feasibility of the immobilization of a mixed microbial culture on brick dust and in calcium alginate beads for the H_2 production was demonstrated by [38]. They found that in a batch culture, cells of the mixed culture in the free state yielded 8.2 L H_2/

mol utilized glucose whereas immobilized cells gave fourfold more H_2 than free bacteria. However, in that study the immobilized cells were stable for only 60 days.

With the aim of encountering more stable supports, [6] used three porous materials (loofah sponge, expanded clay and activated carbon) to allow retention of H_2- producing bacteria (from domestic sewage sludge) within the fixed-bed bioreactors. The authors assessed the carriers for their effectiveness in biofilm formation and H_2 production in batch and continuous cultures. They found that expanded clay and activated carbon exhibited better biomass yields. Then, they used reactors packed with expanded clay and activated carbon (EC or AC reactors, respectively) for continuous H_2 fermentation at a HRT of 0.5–5.0 h. The AC reactor exhibited a better H_2 production rate of 1.32 L/h L, at HRT 1 h. The amount of H_2 produced was promising, yet the H_2 percentages were poor (25–35%). The authors mentioned that when a decline in H_2 production efficiency occurred, the column was heated to 75 °C for 1 h. Nevertheless this treatment was not effective to improve H_2 percentages. This fact could be caused by the high pH (pH 7) since it is not optimum for H_2 fermentation.

Wu et al. [101] immobilized municipal sewage sludge to produce H_2. Cell immobilization was achieved by gel entrapment approaches that were modified by addition of activated carbon (AC), polyurethane (PU) and acrylic latex plus silicone (ALSC). Their results showed that addition of activated carbon into alginate gel (denoted CA/AC cells) enhanced the H_2 production rate over the conventional alginate-immobilized cells and substrate-based yield by 70 and 52%, respectively. For improving the H_2 production activity, the authors repeatedly adapted the immobilized cells to a sucrose medium for a period of time. As a result, a remarkable enhancement in the H_2 production rate with a 25-fold increase for CA/AC and ca. 10 to 15-fold increases for PU and ALSC cells were observed. However, the CA/AC cells produced H_2 only during the first nine runs due to poor mechanical stability and durability. On the other hand, the ALSC cells were found to have better durability but produced slight H_2.

Although the H_2 generation is increased by the use of immobilized-cell systems, the challenge of this technology is the search for supports that fulfill the characteristics described previously, in addition to being stable for long periods of time.

9. Conclusions and perspectives

Anaerobic consortia can be utilized for H_2 production obtaining equal performance in utilizing pure cultures. The main advantage of this process is that organic waste utilization allows working under non-sterile conditions. In this way, production may not need as much steam to achieve sterile/sanitary conditions if the are robust.

However, when non-sterile consortia are employed, H_2 and CO_2 generated are ideal food for H_2-consuming microorganisms, mainly methanogens >autotrophic acetogens, when sulphate and nitrate are negligible. Thus, it is crucial to develop methods to minimize H_2 loss by methanogens in non-sterile systems. Literature shows that in a pH between 5.5–6.5, short hydraulic retention times (HRT > 6 h), heat-shock treatment (80–100 °C for 2–3 h) and acetylene (1%, v/v in the headspace) were efficient for this purpose. Also, the reports show that any one of these methods have to be accompanied by low pH. Long term stability (H_2 production and methanogens inhibiton) and engineering feasibility for large-scale application have to be demonstrated.

Studies on H_2-producers comunities have shown that the predominant genus is *Clostridium* along with *Enterobacter, Thermoanaerobacterium* and *Thermoanaerobacteroids.* There is an association between high H_2 yields and low microbial diversity of communities. Conditions such as low pH, thermophilic temperature, high dilution rate and complex substrates have helped to establish specialized anaerobic consortia. Yet, yields of more than 4 moles of H_2/mol glucose have not been verified from any known microorganism. Therefore, genetic tools can help to develop microorganisms that achieve H_2 yields >4 H_2 mol/mol hexose. Moreover, the microorganisms have to ferment multiple sugars and/or must directly utilize cellulose/hemicellulose (more abundant substrates).

Detailed studies, modeling and engineering of metabolic pathways used in H_2-producing bacteria, including regulation of hydrogenases, are necessary in order to the understand basic chemistry of hydrogenase, overcome the metabolic barrier by manipulating electron flux in H_2 producing organisms, and eliminate unnecesary reactions that use H_2 from glucose to reduce other fermentation products that compete with H_2 production (acids and solvents). These studies are scarce in open literatature. To this end, it is known that the 3D structure of hydrogenase implicated in H_2 evolution from clostridia. The effect of pH, temperature and iron concentration on H_2 production has also been studied although the results are not conclusive.

An important topic is inhibition of H_2 generation by products such as organic acids (acetate and butyrate) and H_2. Accumulation of these end products shift the fermentation from acidogenesis to solventogenesis. Solventogenesis is recognized for generating slight H_2 yields and hydrogensase activities. Triggering for solventogenesis in anaerobic consortia are pH < 5.5, undissociated acids concentrations between 0.3 and 50 mM (variable among species) and H_2 partial pressure >0.5 atm. It is known that high H_2 partial pressure inhibits the hydrogenase activity. Strategies for avoiding H_2 inhibition by high H_2 partial pressure are continuous sparging with inert gas, intermittently vented solid substrate for anaerobic H_2 generation (IV-SSAH) and reduction of H_2 pressure by means of membranes. Up to this point, IV-SSAH seems to be the best option for reducing H_2 pressure and increasing H_2 production performance. However, more engineering advances to reduce H_2 pressure and drive metabolic pathways to acidogenesis are necessary in order to apply the technology to large-scale industrial production.

Finally, in order to obtain a sustainable H_2 production, mixed cultures have been immobilized on diverse materials: brick dust, calcium alginate beads, loofah sponge, expanded clay, activated carbon, activated carbon into alginate gel, and polyurethane and acrylic latex plus silicone. The results were encouraging since H_2 production was several times more than that of free consortia. However, poor mechanical stability and durability were observed. More attention is required in this area.

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